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Experimental *Proteus mirabilis* Burn Surface Infection

Albert T. McManus, PhD; Charles G. McLeod, Jr, DVM; Arthur D. Mason, Jr, MD

• **We established a human burn isolate of *Proteus mirabilis* as an experimental pathogen. Infliction of a nonfatal scald injury (30%) rendered rats highly susceptible to lethal surface infection with this isolate. Dose-response experiments indicated that the lethal inoculation dose (50%) was less than 10^3 organisms per square centimeter. Histopathologically, surface colonization was followed by progressive growth with subsequent invasion of viable tissue. The invasion was not characterized by the perivascular or perineural lesions observed in experimental *Pseudomonas* burn sepsis. Bacteriologic examinations showed moribund animals to be bacteremic with the test strain and to have wound biopsy counts exceeding 10^6 organisms per gram of tissue. The role of bacterial motility as a virulence factor in this surface infection was investigated. Substrains selected for loss of subsurface spreading in soft agar lost virulence. This model of burn infection with a member of the Enterobacteriaceae should be used to evaluate topical and parenteral antimicrobial agents needed for the control of wound infections caused by such agents.**

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It has been approximately 100 years since Koch and his contemporaries founded an experimental approach for establishing the causes of human infectious disease. After isolation of the suspected pathogen, a basic tenet in the design was to establish a model infection with the candidate and to compare animal pathologic course with that in the human disease. This logic was used by Teplitz and colleagues in their classic proof of *Pseudomonas aeruginosa* as a pathogen in *Pseudomonas* burn-wound sepsis.[1,2]

We have attempted to establish an animal model of burn-wound sepsis that would represent a laboratory analogue of infections caused by another group of candidate burn wound pathogens, the Enterobacteriaceae. We have examined human burn isolates from the genera *Enterobacter, Providencia, Klebsiella,* and *Proteus* for experimental pathogenicity in the scalded rat. Our experience has been that most members of the Enterobacteriaceae were not pathogenic for the burned rat. We herein report our findings concerning a human strain of *Proteus mirabilis* that causes fatal experimental burn-wound sepsis.

MATERIALS AND METHODS

Healthy male Sprague-Dawley rats, weighing 330 to 370 g, were used in these studies. In conducting this research, we adhered to the *Guide for the Care and Use of Laboratory Animals,* as promulgated by the Committee on the Revision of the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Research Council.[3] For scalding, the Walker-Mason technique was used.[4] Rats were anesthetized with pentobarbital sodium (25 mg/kg), clipped with an animal clipper with a No. 40 blade, and placed in the Walker-Mason burn mold (Fig 1). Basically, the mold is designed to expose a predetermined area of the rat's total body surface. The template used in these experiments was 149 sq cm, which equates to 30% of the total body surface of a 350-g rat. For burning, the exposed dorsal surface was immersed in boiling water for 10 s. This exposure results in full-thickness injury and does not require parenteral resuscitation for survival to healing.

A human burn-wound isolate, strain 77082234, was used in all experiments. The strain was identified by standard bacteriologic techniques as *P mirabilis.* As an additional identification, the strain was surveyed for enzyme profile using a commercial multiple enzyme assay system (API ZYM). Burned animals were inoculated on the burn wound immediately following injury. Inocula were prepared by diluting overnight tryptic soy broth (TSB) using TSB as the diluent. Inoculation volumes of 1 mL were spread over the 149 sq cm wound.

Fig 1.—Walker-Mason burn mold containing anesthetized rat.

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From the US Army Institute of Surgical Research, Fort Sam Houston, Tex. Dr McLeod is currently with the US Army Biomedical Laboratory, Aberdeen Proving Grounds, Md.

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Reprint requests to Library, US Army Institute of Surgical Research, Brooke Army Medical Center, Fort Sam Houston, TX 78234 (Dr McManus).

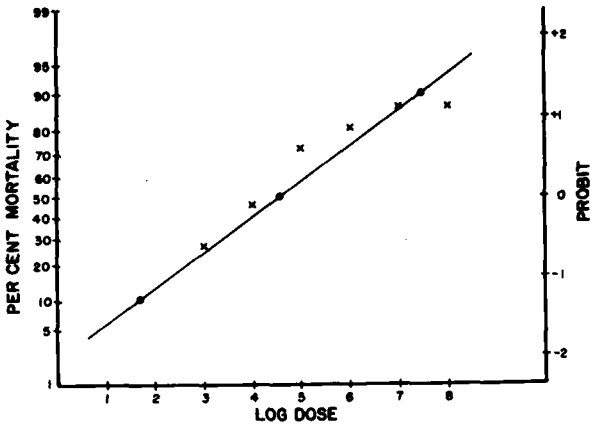

Fig 2.—Effect of total surface inoculum dose on mortality in 350-g rats with 30% full-thickness scalds.

Table 1.—Bacteriologic Findings 24 Hours After *Proteus* Surface Infection

Rat No.	Burn Wound*	Blood Culture	Spleen Culture
1	2.0×10^3	–	–
2	1.4×10^4	+	–
3	3.4×10^2	–	–
4	6.0×10^6	+	–
5	4.0×10^4	–	–
6	6.0×10^4	–	–

*Colony count per gram of flamed tissue.

For serial sacrifice experiments, animals were killed using pentobarbital. Blood culture specimens were taken from the abdominal aorta and cultured in TSB at 37 °C. After incubation for 24 and 48 hours, cultures were plated on tryptic soy agar (TSA). Wound cultures were made from weighed biopsy specimens. Specimens were twice flamed, using 100% isopropyl alcohol. Following flaming, the tissue was ground in TSB using Ten Broeck tissue grinders. Serial dilutions in TSB were made and quantitated by pour plating using TSA at 50 °C. Spleen specimens were cut, weighed, ground, and plated as described for burn biopsy specimens.

The possibility that bacterial motility was a component of this surface infection, which has been documented in *Pseudomonas* burn sepsis, was investigated by examining the virulence of motility mutants.[5] Motility mutants were isolated following exposure of strain 77082234 to 100 μg/mL nitrosoguanidine in 100mM citrate buffer, pH 5.0, at 37 °C for one hour. For mutant isolation, the exposed culture was washed in TSB and incubated for 12 hours at 35 °C in a shaking water bath. The culture was then serially diluted into TSB. Each of the first four dilutions was plated into 50 plates (0.1 mL per plate) and mixed with 10 mL of melted (50 °C), one-third-strength TSA. Following overnight incubation at 35 °C, plates were examined for subsurface nonspreading colonies. Five nonspreading clones were isolated. As a procedural control, five spreading colonies that had been exposed to the same conditions were isolated. The parent isolated nonspreading mutants and control spreading clones were examined for auxotrophy by growth on minimal media.[6] Strains were also examined for loss or gain of standard taxonomic metabolic activities, differences in enzyme profile, and alterations in growth rates in shaking cultures in TSB.

Fig 3.—Gross appearance of wound biopsy specimens at 24 hours after burn. Top specimen is from infected, burned rat. Lower specimen is from burned, noninfected control rat.

Fig 4.—Gross appearance of wound biopsy specimens at 48 hours after burn. Top two specimens are from infected, burned rats, lower specimen is from burned, noninfected control rat.

Relative virulence of the isolated mutants and isolated control strains was assessed by burn-surface inoculation (10^8 colony forming units [cfu]) of scalded rats. As an additional test, nonspreading mutants were injected below the eschar with the intent of assessing virulence without the requirement of surface invasion.

The strain was examined for in vitro antibiotic sensitivity. Several appropriate antibiotics were tested for activity in fatally infected rats. Treatment was initiated six hours after burning and infection with 10^7 cfu. Topical chemotherapy was attempted with 1% sulfadiazine silver and with 11.2% mafenide acetate. Parenteral antibiotics used were gentamicin sulfate, 4 mg/kg intramuscularly (IM), amikacin sulfate, 8 mg/kg IM, and carbenicillin disodium, 500 mg/kg intraperitoneally. Animals were treated twice per day for ten days and observed for 21 days.

RESULTS

Initial experiments with strain 77082234 showed that inoculation of 1 mL of an overnight culture onto rat burn wounds resulted in all rats dying within 72 hours. The results of dilution of the inoculum are presented (Fig 2) as the probit regression of cumulative mortality as a function of inoculation dose. From this figure, the 50% lethal dose

Fig 5.—Section of burned infected wound at 48 hours (hematoxylin-eosin, ×25).

Table 2.—Bacteriologic Findings 48 Hours After *Proteus* Surface Inoculation

Rat No.	Burn Wound*	Blood Culture	Spleen Culture*
1	3.0×10^7	+	1.3×10^2
2	1.2×10^6	+	6.0×10^4
3	1.4×10^6	+	10^1
4	1.0×10^7	+	1.4×10^2
5	3.1×10^6	+	–
6	4.7×10^6	+	2.7×10^1
7	2.1×10^7	+	4.3×10^2
8	1.5×10^6	+	3.2×10^2

*Colony count per gram of flamed tissue.

is about 600 organisms per square centimeter of burn wound.

Serial sacrifice experiments with rats inoculated with 10^6 cfu showed progressive infection. Biopsy specimens taken 24 hours after infection showed no gross difference in appearance (Fig 3). Bacteriologic findings showed biopsy specimens to contain approximately 10^4 organisms per gram of tissue (Table 1). Two of the rats had positive blood cultures, but spleen cultures were negative, indicating the

Fig 6.—Section of burned infected wound at 48 hours (tissue Gram's stain, ×100). Arrows indicate Gram-negative organisms.

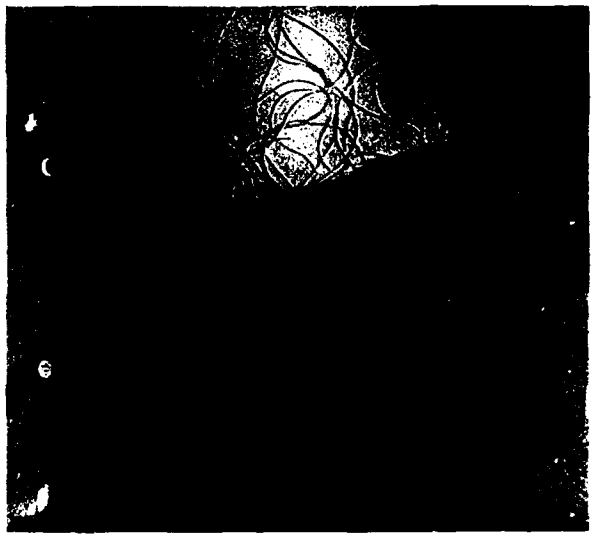

Fig 7.—Electron photomicrograph of strain 77082234 (chromium shadowed, ×19,500).

presence of fewer than 10 organisms per gram of tissue. At 24 hours after burning and infection, the rats showed no clinical signs of sepsis.

Rats examined at [illegible]fter burning and infection were weak and letha[illegible] wounds appeared raised and were rigid to the touch. [illegible]sy showed gross thickening (Fig 4). Histopathologic examination showed suppuration and edema (Fig 5). Tissue Gram's staining revealed massive accumulation of Gram-negative organisms invading viable subdermis and fat (Fig 6). Quantitative bacteriologic examination showed more than 10^6 organisms per gram of flamed tissue (Table 2). Septicemia was demonstrable, all rats having positive blood cultures, and all but one rat had positive spleen cultures.

Strain 77082234 has peritrichous flagellation (Fig 7) and demonstrates swarming (Fig 8). Following nitrosoguanidine exposure, all mutants that lost subsurface spreading

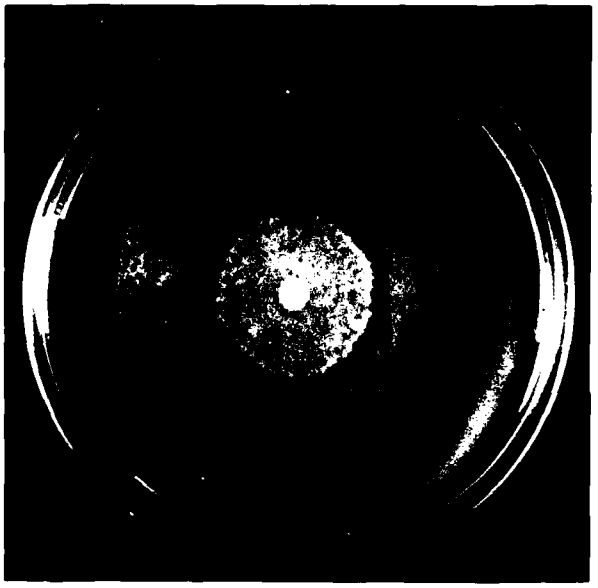

Fig 8.—Swarming behavior of strain 77082234 following central inoculation of 100-mm Petri dish of tryptic soy agar (incubation 18 hours at 37 °C).

ability also lost the ability to swarm on the surface. Of the five nonspreading mutants, three lost flagellation and two did not. Metabolically, the five nonspreading clones and the five spreading control clones remained as the parent strain, prototrophic. Enzyme profiles also were unchanged, with all strains showing high levels of alkaline phosphatase, leucine aminopeptidase, trypsin-like protease, acid phosphatase, and phosphoamidase. All strains produced lesser activities of esterase lipase, valine aminopeptidase, and nonspecific esterase. Nonspecific lipase, chymotrypsin-like protease, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase activities were not detected. Growth rates were also similar between strains.

Testing for relative virulence of *P mirabilis* motility mutants gave the following results:

Strain	Mortality
Parent (surface)	20/20
Nonmotile (surface)	13/80
Nonmotile (injected)	36/40
Motile control (surface)	25/30

Nonmotile mutants were less virulent when inoculated on the surface than were the other three classes ($\chi^2 = 93.189$, $df = 1$, $P < .01$). The other classes were not different from one another ($\chi^2 = 1.34$, $df = 2$, not significant).

Strain 77082234 was sensitive in vitro to aminoglycosides, sulfonamides, semisynthetic penicillins, and chloramphenicol. Treatment of fatally infected rats with gentamicin, amikacin, carbenicillin, and sulfadiazine silver was effective. Mafenide acetate was not effective in this model (Table 3).

Table 3.—Chemotherapy for Experimental *Proteus mirabilis* Burn-Wound Sepsis

	Mortality						
	Day After Burn						
Treatment*	1	2	3	4	5	6	Total
Gentamicin sulfate, 4 mg/kg IM	0	0	0	0	0	0	0/10
Amikacin sulfate, 8 mg/kg IM	0	0	0	0	0	0	0/10
Carbenicillin disodium, 500 mg/kg IP	0	0	0	0	0	0	0/10
Sulfadiazine silver, 1% topical	0	0	0	1	0	0	1/10
Mafenide acetate, 11.2% topical	0	0	6	1	0	2	9/10
Infected control	1	3	4	2	0	0	10/10

*Animals were treated twice per day starting six hours after infection. IM indicates intramuscular; IP, intraperitoneal.

COMMENT

Proteus mirabilis strain 77082234 has been found to cause an invasive surface infection of the burned rat. The temporal course of the disease is a continuum from surface contamination to heavy surface growth and microinvasion at 24 hours, systemic toxicity at 40 hours, full invasion with septicemia at 48 hours, and death at 48 to 96 hours. The basis of the toxicity is unknown, though several possibilities exist. One is that the metabolic products of urea metabolism, namely ammonium ion, may be toxic as described in experimental pyelonephritis.[7,8] A second possibility is that the toxicity may be the result of host responses to other absorbed bacterial products such as proteolytic enzymes and lipopolysaccharide. We have not as yet gathered enough data to posit a specific toxic mechanism in this model, but we do believe that toxicity is an important component.

Invasive infection is concurrent with toxicity. As a preliminary approach to examine the role of tissue invasiveness, we tested the hypothesis that active bacterial motility is required for virulence. As noted, strains that had lost both the ability to spread in soft agar and surface swarming were attenuated in terms of virulence in this model. Though these data might imply that loss of motility was associated with loss of production of toxins or other pathogenic mechanisms, it seems more reasonable to assume that loss of the ability to penetrate and spread in the burn wound limited the expression of pathogenic mechanisms. When nonspreading strains were injected below the barrier of the devitalized wound, they were as virulent and toxic as the parent strain. This supports the concept that active motility is an invasion and spreading factor in this model.

Aside from establishing pathogenicity of *P mirabilis* in an experimental model, our intent was to provide a model representing burn-surface infection with one of the Enterobacteriaceae. Such a model would allow laboratory evaluation of chemotherapy intended for such organisms. Strain 77082234 was found to be sensitive in vitro and in vivo to several clinically usable antibiotics. We have

initiated efforts to modify the sensitivity pattern of this strain by introduction of specific R factors. With the development of genetically identifiable resistances, this model may be useful in evaluation of experimental antibiotics designed to overcome existing bacterial resistance mechanisms.

William J. Northam, Peter A. Dorsaneo, and Paulette Langlinais, MS, provided technical support in conducting the study.

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Discussion

RICHARD F. EDLICH, MD, Charlottesville, Va: *Proteus* species have been incriminated in a variety of infections, including burn-wound sepsis, urinary tract infections, wound infections, and intra-abdominal infections. They are, in fact, the second most common cause of urinary tract infections. The investigators' approach to the study of the pathogenesis of *P mirabilis* burn-wound infection is reminiscent of that employed in the investigations of the mechanisms of pathogenicity of *Proteus*-induced urinary tract infection (*J Bacteriol* 1960;80:171-179). They account for the susceptibility of the kidney to *Proteus* on the basis of its powerful urease. The urease splits urea into carbon dioxide and ammonium. The ammonium released by the bacterial enzyme alkalinizes the urine and lowers the resistance of the tissue by inactivating complement. Alkalinization disrupts the uroepithelium and allows the organism to penetrate the renal parenchyma and cause tissue damage. Interestingly, the importance of bacterial motility was not elucidated clearly in these experimental studies of the pathogenesis of urinary tract infection.

In this study, bacterial motility as measured by subsurface spreading in soft agar appears to be a requirement for burn-wound sepsis. These findings are similar to previous studies of *Pseudomonas aeruginosa* burn-wound sepsis in which the authors, Dr McManus and colleagues, again stressed the importance of motility (*Burns* 1980;6:235-239). This unifactorial concept is correct unless we assume that agar spreading is linked with other virulence characteristics of the parent organism or that mutagenesis resulted in loss of other unrelated virulence-linked markers that were picked in the nonspreading strains and not in the motile strains. Though the authors may believe that these alternatives are unreasonable, I would submit that they are not. The authors have identified an important association between motility and virulence, but they have not conclusively demonstrated a cause and effect. Their failure to elucidate the pathogenesis of *P mirabilis* burn-wound infection is not unique, however, as our understanding of the pathogenesis of most infections is still in its infancy.

The authors conclude that *P mirabilis* infection can be controlled with appropriate chemotherapy. The selection of the appropriate antibiotic was based on the results of the antibiotic disk sensitivity test. Systemic treatment of the infection was restricted only to the antibiotics to which the strain showed sensitivity. Antibiotics to which the strain displayed resistance were not tested in this model. Consequently, it is impossible to conclude that the infection can only be controlled by the appropriate antibiotic.

Finally, the raison d'être for the success of topical antibiotics is unclear. Topical application of sulfadiazine silver was effective, whereas mafenide acetate had no value. It might be interesting to relate these therapeutic outcomes in vivo to standardized antimicrobial cream sensitivity tests in vitro.

E. PATCHEN DELLINGER, MD, Seattle: The use of nitrosoguanidine to get a nonmotile *Proteus* is one way to approach the problem of whether or not motility is important. It is a kind of a gross technique microbiologically and will commonly result in a lot of other mutations that may not be detected.

With the new molecular genetics available, a good way to look at this problem would be to try to isolate the genetic material for motility and to insert that into a nonmotile, nonpathogenic strain and examine its pathogenicity. This is being done a lot with *Escherichia coli* these days in different models of virulence.

DR MCMANUS: I must stress that motility was the only bacterial function that we have looked at so far, but I certainly realize that there are many other possibilities.

We are isolating urease-negative clones and protease-negative clones of this strain, and their virulence is being evaluated. By changing the urease, we may be changing a taxon.

In answer to Dr Dellinger's question, I realize the limitations of nitrosoguanidine. I cannot say that no other function has been changed, but the fact that these mutants have remained prototrophic, I think, suggests that it may not have been as tough on them as it could have been.

As far as genetic manipulation, rather than chemical mutagenesis, what we are trying to do now is introduce an ampicillin resistance gene that behaves as a genetically transposable element into some of the motility genes, thereby avoiding the whole question of chemical mutagenesis. We have not been able to do this yet, but I think eventually we will.

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